REMARKS

Claims 1-10, 18-34, and 39-51 are pending. Applicants acknowledge that the restriction requirement has been deemed final. Accordingly, claims 2-3, 5, 7-9, and 45-51 are withdrawn from further consideration as being drawn to a non-elected invention. Claims 1, 4, 6, 10, 18-34, and 39-44 are therefore under examination with respect to the elected species of a histamine or serotonin binding protein which has a first binding site and a second binding site; a histamine or serotonin binding compound which comprises a synthetic compound; a histamine or serotonin binding compound that is genetically fused to one or more peptides; cysteinyl leukotriene; SEQ ID NO: 4; the species of isoleucine at position I, tryptophan at position II, aspartate at position III, and glutamate at position IV with regard to the first binding site; and the species of isoleucine at position I, tryptophan at position II, glycine at position III, and aspartate at position IV with regard to the second binding site. Claims 8, 9, 19, 33, 39, 40, 41, 43, and 45-51 are canceled herein without prejudice. Claims 11-17 and 35-38 were previously canceled. Claims 1, 4, 6, 10, 18, 20-30, 34, 42, and 44 are amended herein to more clearly set forth aspects of the invention. New claims 52 and 53 are submitted herewith. Accordingly, claims 1, 4, 6, 10, 18, 20-30, 34, 42, and 44 as amended and dependent claims therefrom, and new claims 52-53 are under consideration.

Any amendment is not to be construed as abandonment of any subject matter of the application as originally filed. Accordingly, it is to be understood that Applicants reserve the right to reintroduce subject matter deleted from the application by the foregoing amendments and to file one or more divisional, continuation, and/or continuation in part applications directed to such subject matter.

Support for the amendments to the claims is found throughout the specification and in the original claims. Specifically, support for amendments to claims 1, 4, 6, 10, 18, 20-30, 34, 42, and 44 is presented, for example, at page 22, line 27 through to page 24, line 29, wherein support for the term "isolated" is found. Support for amendments to claims 1, 4, 6, 10, 18, 20-30, 34, 42, and 44 is presented in the original claims and in the specification, for example, at page 5, lines 10-11, wherein support for SEQ ID NO: 4 being referred to as a protein is found. Support for amendments to claims 1 and 4 is found in original claims 1 and 4, and at page 6, lines 13-20, wherein support for

"functionally equivalent complementarity of shape" is found. Claims 4 and 6 are amended to clarify the numbering of the amino acid residues tyrosine (claim 4) and cysteine (claim 6) of D.RET6 as referred to therein. Support for amendments to claims 4 and 6 is presented in the specification, for example, in Figure 4, wherein the amino acid sequence of full length D.RET6 protein (SEQ ID NO: 4) is presented. The full length D.RET6 protein includes a 17 amino acid N-terminal leader sequence, which was not accounted for in original claims 4 and 6. The numerical delineations of the recited tyrosine and cysteines of D.RET6 have, therefore, been shifted by 17 residues to clarify this issue and maintain consistency throughout the specification and the claims. Support for amendment to claim 20 is found, for example, at page 11, lines 2-5, wherein the term "toxin or bioactive molecule " is described. Support for amendment to claims 25 and 26, is found, for example, at page 10, lines 26-27 and page 11, lines 9 to 30. Support for amendments to claims 34 and 42 is presented in original claims 34 and 42 and, for example, at page 1, lines 7-10; at page 1, line 29 through to page 2, line 10; at page 2, line 30 through to page 3, line 29; and page 13, lines 19-26, wherein support for methods of using vasoactive amine binding compounds, proteins, and compositions for the treatment of diseases or pathological effects resulting from the activity of vasoactive amines is found; and at page 4, lines 1-4, wherein support for an antagonistic agent for the treatment of allergic reactions is found. No issue of new matter is introduced by these amendments.

New claims 52 and 53 are submitted herewith. Support for new claims 52 and 52 is found throughout the specification and in the original claims. Specifically, support for new claims 34 and 42 is found in original claims 34, 42, and 44 and, for example, at page 1, lines 7-10, at page 1, line 29 through to page 2, line 3, and page 13, lines 19-26, wherein methods of using serotonin binding compounds, proteins, and compositions for the treatment of diseases or pathological effects relating to the activity of serotonin are described. No issue of new matter is introduced by this amendment.

The specification is amended herein include required sequence identifiers, an abstract on a separate sheet of paper, and to reflect Applicants clarification of the amino acid residue positions in SEQ ID NO: 4 as presented in the amendment of 09 September 2003. Support for the amendment to the specification is found in the specification as

filed, particularly in Figure 4, and in the original claims. To be consistent with the amendment of 09 September 2003, the specification has been amended to clarify the numbering of the amino acid residues at positions I-IV of the first binding site of D.RET6, thereby replacing recited residues "122, 54, 50 and 95" with residues "139, 71, 67, and 112". The discrepancy in numbering with regard to positions I-IV of the first binding site, which amounts to a consistent differential of 17 amino acids, stemmed from the difference in length observed between the full length protein, which comprises an N-terminal leader sequence, and the mature protein, from which the leader sequence has been processed or removed. SEQ ID NO: 4 which is presented in Figure 4 depicts the amino acid sequence of full length D.RET6 protein. The numerical delineations of positions I-IV of the first binding site of D.RET6 have, therefore, been shifted by 17 residues to clarify this issue. No issue of new matter is introduced by these amendments.

Claims 1, 4, 6, 27, and 32 are objected to for reciting non-elected groups and species. Claims 6, 10, 18-21, 25-36, 28-30, 32-34, and 39-44 are objected to for claiming dependency from claims which are withdrawn. Applicants have amended claims 1, 4, 6, 10, 18-21, 25-30, 32-34, and 39-44 to address these issues, thereby rendering moot the objection to these claims. Support for these amendments in found in the specification as filed and in the original claims. No issue of new matter is introduced by these amendments.

The Examiner has referred to the following art, which is made of record, but not relied upon, and considered pertinent to Applicants' disclosure: Paesen et al. (Biochim Biophys Acta 1309(1-2):9-13, 1996); Paesen et al. (Biochim Biophys Acta Oct 18; 1482(1-2):92-101, 2000); and Paesen et al. (Mol Cell 3(5):661-71, 1999); and Sangamnatdej et al. Insect Molec Biol 11(1):79-86, 2002). Applicants are familiar with these references and confirm that these references do not impact the patentability of the present invention.

Rejections under 35 USC § 101

Claims 1, 4, 6, 10, 18-34, and 39-44 stand rejected under 35 U.S.C. § 101 for allegedly being drawn to non-statutory subject matter. Claims 1, 4, 6, 10, 18, 20-30, 34, 42, and 44 and dependent claims therefrom are amended herein to indicate that the claims

are directed to isolated histamine or serotonin binding compounds. Applicants, therefore, believe that the basis of this rejection is addressed by this amendment, and respectfully requests that the rejection of claims 1, 4, 6, 10, 18-34, and 39-44 be withdrawn.

Claims 34 and 39-43 are rejected under 35 U.S.C. § 101 for allegedly being directed to a claimed process in the absence of recited steps involved in the process. Claims 39 and 40 are canceled herein, thereby obviating any rejection of these claims. Claims 34 and 42 are amended to recite steps involved in the claimed process.

Rejections under 35 USC § 112

Claims 1, 4, 6, 10, 18-24, and 29 have been rejected under 35 USC § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one of skill in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. In view of the amendments to the claims, and corroborative evidence combined with Applicants' arguments presented herein, the rejection, as it applied to claims 1, 4, 6, 10, 18-24, and 29, is respectfully traversed.

The Examiner appears to have largely based the rejection of these claims on recitation of the phrase "and functional equivalents thereof". The claims are amended herein to remove reference to such functional equivalents. The Examiner has also indicated that the specification is enabling for a binding protein comprising the amino acid sequence of SEQ ID NO: 4 possessing the claimed properties, but appears to consider recitation of a "binding compound", rather than a "binding protein" or the like, lacking in support. Although Applicants maintain that the claims are clearly directed to a histamine or serotonin binding compound that comprises the amino acid sequence of SEQ ID NO: 4 with the recited features and properties, Applicants have amended claims 1, 4, 6, 10, 18, 20-30, 34, 42, and 44 to recite "binding protein".

With regard to the Examiner's comments pertaining to evidence demonstrating that serotonin binds to the D.RET6 protein comprising SEQ ID NO: 4, Applicants respectfully direct the Examiner to Example 3 of the filed application, wherein results are presented showing that the presence of serotonin dramatic increases the affinity of D.RET6 for histamine. In that the histamine binding assay described in Example 3 is a

precisely controlled *in vitro* experiment with a highly restrictive number of assay components added, including essentially only two active components, D.RET6 and histamine, a skilled practitioner would reasonably interpret the demonstrated effect of serotonin in such an assay to be the result of a direct effect of serotonin binding.

Such an interpretation has been affirmed by the present inventors who have shown that D.RET6 binds serotonin *in vitro*. See Exhibit A. As shown in the Table presented in Exhibit A, the amount of detectable serotonin remaining in supernatants following incubation with various concentrations D.RET6 was reduced to below measurable levels. These data suggest that D.RET6 binds to serotonin and sequesters the serotonin, thereby masking the detectable levels of serotonin in the supernatant.

The Examiner also appears to allege that the specification does not properly support the feature that *"residues I to IV are positioned substantially the same as residues 139, 71, 67 and 112 in SEQ ID NO.4 ..."*. Specifically, the Examiner states that the specification only discloses the binding protein comprising the amino acid sequence of SEQ ID NO: 4 and its characterization and appears to indicate that Applicants should be limited accordingly.

When determining the scope of protection, it is not proper to limit Applicants to the specific examples presented that illustrate a general phenomenon. Applicants should be allowed to cover all obvious modifications, equivalents and uses of that which they have described. Clearly, it is essential for Applicants to be allowed an appropriate level of breadth when casting their claims, as failure to have a broad claim encompassing more than just the exact sequence of the specific protein would be tantamount to seizure of an invention discovered by Applicants.

For this reason, the USPTO routinely grants claims that extend beyond the specific protein(s) that are disclosed in the specification so that the claims cover obvious equivalents of the specific protein(s). In this regard, the Examiner is referred to Applicants' earlier U.S. Patent No. 6,617,312 which formed the basis of a double patenting objection. The claims of this patent are not limited to a specific protein sequence but extend to homologous proteins having a specified sequence motif.

In the present application, the Applicant has disclosed which residue positions of the D.RET6 protein (SEQ ID NO: 4) are important for the high affinity binding to

serotonin and histamine and also which amino acid residues may be located in these positions. Once the skilled person is equipped with this information, routine techniques may be employed to identify other compounds having the properties set forth in the claims. Considerable guidance is included in the specification on how to prepare such compounds. For instance, see page 6, lines 21 to page 7 line 8 of the specification which states:

> *"Current methods of generation of compounds with affinity for a molecule of interest have been until recently relatively primitive. The notion of combinatorial chemistry and the generation of combinatorial libraries has, however, developed at great speed and facilitated the rational design and improvement of molecules with desired properties. These techniques can be used to generate molecules possessing binding sites identical or similar to those of the histamine or serotonin binding sites identified herein."*
>
> *Such compounds may be generated by rational design, using for example standard synthesis techniques in combination with molecular modelling and computer visualisation programs. Under these techniques, the "lead" compound with a similar framework to the histamine or serotonin binding site is optimised by combining a diversity of scaffolds and component substituents.*
>
> *Alternatively, or as one step in the structure-guided design of a molecular entity, combinatorial chemistry may be used to generate or refine the structure of compounds that mimic the histamine or serotonin binding site of histamine or serotonin binding compounds by the production of congeneric combinatorial arrays around a framework scaffold. These steps might include standard peptide or organic molecule synthesis with a solid-phase split and recombine process or parallel combinatorial unit synthesis using either solid phase or solution techniques (see, for example Hogan, 1997 and the references cited therein).*

As will clearly be appreciated from this passage, modern techniques have vastly simplified the task of identifying compounds having the claimed properties. Further guidance on how to prepare the compounds of the invention is provided on page 7, line 19 to page 8, line 11 and in Example 4, beginning at page 28.

The Examiner has cited two references (Wells et al. 1990, Biochemistry 29:8509-8517 and Ngo et al. 1994, The Protein Folding Problem and Tertiary Structure Prediction, pp. 492-495) that allegedly support the Examiner's position with regard to amino acid

residues that are critical to the structure/function of a protein and limited tolerance for substitutions at such sites. The Examiner also maintains that Applicants have allegedly provided little or no guidance beyond presentation of sequence data pertaining to the D.RET6. Applicants strenuously disagree with the Examiner's position with respect to this contention. As detailed in the specification and herein, the field of computer-assisted or *in silico* analyses of amino acid sequences has advanced exponentially since these rather antiquated references were published. Applicants, therefore, assert that the state of the present art is not well reflected in the commentary of references that are fourteen and ten years old, respectively.

Moreover, Applicants assert that teachings presented in the Specification go far beyond that of a presentation of the nucleic and amino acid sequences of D.RET6. Critical residues implicated in vasoactive amine binding are identified and discussed throughout the Specification. The Examiner's particular attention is directed to Figure 22, wherein an alignment of amino acid sequences of D.RET6 and related family members is presented and such critical residues are indicated. It should, furthermore, be stressed that the inventors of the present invention have demonstrated experimentally that D.RET6 and its structurally related family members are functionally related as well, in that they are all capable of binding vasoactive amines. In addition to the above compelling evidence that attests to a substantial degree of guidance presented in the specification, the inventors have also established the crystal structure of one of the structurally and functionally related family members, namely FS-HBP2. See Example 4, starting at page 28, line 15 and Figures 18-21. Summaries pertaining to the overall structure and unique features of FS-HBP2 are found at page 31, line 31 through to page 33, line 7. Notably, it would be a matter of routine practice to perform computer modeling to predict the crystal structures of the other related vasoactive amine binding protein family members (e.g., D.RET6) based on this solved crystal structure. Additional findings directed to addressing the relative affinities of the two identified histamine binding sites and conformational ramifications of histamine binding are also presented at page 33, line 8 through to page 34, line 30. Guidance relating to sequence variation observed among the vasoactive amine binding family members and the implications of such variation is also found at page 34, line 31 through to 35, line 18. The detailed

information pertaining to the pockets that bind vasoactive amines is particularly revealing with respect to guidance as to which residues therein can tolerate substitutions and how spacing between critical residues may be varied without adversely affecting the binding pocket.

In view of the above, Applicants assert that the Specification presents substantial teachings pertaining to primary amino acid sequence, biological function and identification of critical residues implicated in this function, and three-dimensional crystal structure that enable an ordinarily skilled artisan to practice the claimed invention.

The Examiner has indicated that the presence of the specified amino acids at the specified positions may not be sufficient for the desired binding activity because:

> *"the art recognizes that function cannot be predicted from structure alone".* (page 8, line 2)

The Examiner is, of course, correct that not all proteins that satisfy the structural (sequence) requirements of the claim will necessarily bind to histamine or serotonin with the described affinity. It is for precisely this reason that Applicants have adopted the "structure + function" language of the claims, such that only a protein that satisfies the sequence requirements of the claims and binds to histamine or serotonin with very high affinity is embraced. Accordingly, proteins that satisfy the sequence requirements of the claims but which do not bind with high affinity to histamine or serotonin are not embraced by any of the claims.

In order to ascertain the binding properties of a putative equivalent of the D.RET6 protein of SEQ ID NO: 4, histamine and serotonin binding assays may be employed by the skilled person. Histamine binding assays are described in the specification (see pages 26 and 27) and were routine at the priority date of the present application. The skilled practitioner would thus have no difficulty in identifying proteins that fall within the scope of the claims and thus no information is missing from the specification that would preclude such a skilled practitioner from practicing the invention across the entire scope that is presently claimed.

Thus, Applicants assert that the specification is enabling for a histamine or serotonin binding compound capable of binding to histamine or serotonin with a

dissociation constant of less than 10^{-7}M and which has a binding site comprising amino acid residues isoleucine at position I, tryptophan at position II, aspartate at position III and glutamate at position IV, wherein residues I to IV are positioned at residues 139, 71, 67 and 112 in SEQ ID NO: 4 or are positioned in a functionally equivalent complementarity of shape.

The Examiner has also cited numerous references supportive of the contention "that function can not be predicted from structure alone". The list of references includes the following: Bork (2000, Genome Research 10:398-400); Skolnick et al. (2000, Trends in Biotech. 18(1):34-39); Doerks et al. (1998, Trends in Genetics 14:248-250); Smith et al. (1997, Nature Biotechnology 15:1222-1223); Brenner (1999, Trends in Genetics 15:132-133); and Bork et al. (1996, Trends in Genetics 12:425-427). These references largely pertain to genome wide sequence analysis and functional annotation of newly identified nucleic acid sequences. Pitfalls of such functional annotation in the absence of additional corroborative data are emphasized in these references. Applicants assert that these references have little bearing on the present invention. This assertion is underscored by the teaching and guidance presented in the Specification which sets forth the primary amino acid sequence of D.RET6 and other related vasoactive amine binding family members and a sequence alignment of these related proteins, identifies and characterizes biological functions possessed by this family of proteins (i.e., the ability to bind vasoactive amines), delineates critical residues implicated in these functions, and presents three-dimensional crystal structures of one of the related family members in various states complexed to either one or two molecules of a vasoactive amine. It is respectfully submitted that the Examiner appears to have overlooked the fact that Applicants have not only presented experimental evidence demonstrating function, but have also determined the crystal structure of a vasoactive amine binding protein family member and identified the vasoactive amine binding pockets therein. Thus, Applicants assert that the presently claimed invention is amply enabled by the Specification and certainly does not suffer the "pitfalls" described in the above-listed references.

In view of the above, applicants contend that the rejection of claims 1, 4, 6, 10, 18-24, and 29 under 35 U.S.C. §112, first paragraph, is rendered moot and respectfully request that the rejection be withdrawn.

Claims 25-28, 30-34, and 39-44 stand rejected under 35 USC § 112, first paragraph, on the basis, as is understood, of an alleged lack of enablement. In view of the amendments to the claims and Applicants' arguments presented herein, the rejection, as it applied to claims 25-28, 30-34, and 39-44, is respectfully traversed.

The Examiner appears to suggest that claims relating to a histamine or serotonin binding molecule fused to a carbohydrate moiety, another protein, or a toxin are not enabled by the specification and that undue experimentation would be required by a skilled practitioner to fuse **all** possible histamine/serotonin binding molecules with any carbohydrate, protein or toxin and screen them for activity. To begin, Applicants would respectfully direct the Examiner's attention to the fact that the claims are directed to a histamine or serotonin binding protein comprising SEQ ID NO: 4 and having the recited properties. The claims are not directed to **all** histamine or serotonin binding proteins.

Moreover, and contrary to the Examiner's position, the rationale behind fusion of a histamine or serotonin binding protein with a further moiety is not to create a new activity brought about by the association of the two moieties. Rather, the rationale is to bring together two moieties in a fusion protein (the binding protein plus the additional moiety), such that each moiety contributes the functional activity/activities exhibited when in isolation (i.e., when not fused to another moiety) and the fusion protein exhibits a combination of these desired activities. This is clearly evident from page 10, line 26 to page 11, line 30.

Techniques for the preparation of fusion proteins are well documented in the art and the preparation of such fusion proteins is a matter of routine practice. Hence, the assertion that the skilled person would not be able to fuse two moieties together so that each moiety acts in its usual manner is completely misplaced.

The Examiner has also alleged that the specification does not teach how to use a histamine or serotonin binding compound in the treatment of a condition or disease in an animal because no working examples of this are disclosed. The Examiner appears to be suggesting that *in vivo* evidence must be disclosed to support a claim relating to the therapeutic use of a compound. This position is considered unreasonable.

As the Examiner must appreciate, sometimes it is simply not possible to wait until *in vivo* data are available before filing a patent application. Were the test applied by the

Examiner in this case to be the approved threshold for justifying a claim to the medical use of a particular compound, very few, if any, method of treatment claims would ever be granted. This is, of course, not the case. Provided that there is no technical information missing from the specification for the skilled reader to be able to practice the invention, *i.e.* to use the claimed product as a pharmaceutical agent, the specification should be regarded as being enabling.

The Examiner appears to allege that the specification is not enabling because it does not teach the skilled artisan the optimal dosage, duration and mode of administration of any histamine or serotonin binding compound. Again the Examiner appears to be placing an unreasonable burden on Applicants and it is noted that the United States Patent and Trademark Office routinely grants patents for therapeutic methods where the optimal dosage, duration and mode of administration are not disclosed in a comprehensive manner. In any event, possible modes of administration are outlined in the specification at page 14, lines 3 to 10 and the elucidation of optimal dosages and duration of treatment is well within the abilities of a skilled person.

The Examiner also alleges that the claimed methods may not necessarily treat allergy or inflammation. The Examiner has, however, failed to provide any reasons for alleging that the claimed methods may not be capable of treating inflammation. As is evident from the specification (e.g. page 1, second paragraph) the link between histamine and serotonin release and inflammation is well documented. Accordingly, compounds which bind to histamine and serotonin and make it unavailable for binding to histamine and serotonin receptors are expected to be beneficial in the treatment of inflammation. Moreover, evidence of the histamine binding properties of D.RET6 is presented in Example 3 of the application as filed and additional data demonstrating the serotonin binding properties of D.RET6 *in vitro* are shown in Exhibit A.

Corroborative evidence pertaining to the efficacy of two of the related, non-elected claimed compounds to treat inflammation (FS-HBP2 (SEQ ID NO: 2) and MS-HBP (SEQ ID NO: 3)) is also presented herein in Exhibits B and C. The efficacy of using vasoactive amine binding proteins FS-HBP2 and MS-HBP1 for treating two types of allergic reaction; allergic conjunctivitis and allergic rhinitis (hay fever) is clearly demonstrated in results shown in Exhibit B:

- Example 1 describes a study pertaining to the efficacy of various concentrations of an ophthalmic concentration of the vasoactive amine binding protein FS-HBP2 in counteracting the effects of a pro-inflammatory compound administered to a rabbit. It concludes that topical pre-treatment with FS-HBP2 has a significant effect in reducing the build-up of mucus in the eye and in reducing the change from baseline redness.

- Example 2 further investigates the effect of pre-treatment with FS-HBP2 and concludes that there is a protective effect conferred by such pre-treatment, as reflected by a substantial reduction in the degree of eye redness resulting from subsequent application of a pro-inflammatory compound. This effect has a significant duration (8 hours).

- Example 3 is a study of the effect of a second vasoactive amine binding protein, MS-HBP1, in counteracting the effect of intra-nasal challenge with histamine. The study concludes that this protein causes a four-fold shift in the dose-response to histamine and is, therefore, likely to be therapeutically-effective in the prevention or treatment of allergic rhinitis.

Additional evidence of the effectiveness of vasoactive amine binding proteins is shown in Exhibit C. Example 1 therein presents results relating to the efficacy of FS-HBP2 (referred to in Exhibit C as EV131) for the treatment of allergic asthma and a similar condition, designated acute respiratory distress syndrome (ARDS). Example 2 of Exhibit C imparts results that demonstrate the utility of FS-HBP2 (EV131) as a therapeutic agent for the treatment of allergic conjunctivitis.

Thus, Applicants assert that there is extensive evidence showing that vasoactive amine binding proteins disclosed in the application that are capable of binding histamine and serotonin are useful in treating a disease or condition in an animal and in particular are useful in treating allergy and inflammation. Given that Applicants have demonstrated that D.RET6 binds histamine and serotonin, coupled to the evidence that other related

histamine and serotonin binding molecules can be used successfully to treat inflammation and allergies *in vivo*, there is a reasonable expectation that D.RET6 will also prove to be an effective agent for treating allergy and inflammation.

The Examiner has indicated that aspects of the invention directed to using a histamine or serotonin binding compound to detect and/or quantify histamine in a variety of test sources may be unpredictable. Although Applicants maintain that such an application would be well within the capabilities of a skilled practitioner, claims directed to this aspect of the invention are canceled herein without prejudice.

Furthermore, it the Examiner's position that the use of a histamine or a serotonin binding compound as a vaccine would be adversely impacted by problems associated with administering a subunit vaccine to humans and animals. In response, although Applicants assert that there is no evidence to suggest that such problems would be applicable to compounds of the present invention, claims directed to this aspect of the invention are canceled herein without prejudice.

The Examiner has also suggested that aspects of the invention directed to using a histamine or serotonin binding compound to prevent inflammation or allergic reaction in an animal are allegedly not properly supported. Applicants assert that the specification imparts ample guidance with which a skilled artisan can practice the present invention. As described herein above, the link between the release of histamine and serotonin and inflammation is well established and described in the specification. Applicants have demonstrated the histamine and serotonin binding properties of D.RET6, as revealed by results presented in Example 3 of the filed application and in Exhibit A. It thus follows that histamine and serotonin binding compounds capable of interfering with the ability of these vasoamines to engage their respective receptors can be used to advantage in the prevention of inflammation and allergic reaction. Applicants assert that D.RET6 fulfills the criteria set forth for such a preventive agent. Moreover, the efficacy of related compounds FS-HBP2 and MS-HBP in the prevention and treatment of inflammatory conditions in animal model systems is demonstrated by results presented in Exhibits B and C.

Examples 1 and 2 of Exhibit B demonstrate that pre-treatment of rabbit eyes with FS-HBP2 prevents and/or reduces many symptoms characteristic of conjunctivitis. The

efficacy of such treatment is measured qualitatively and quantitatively by reduced redness and inflammation of the eyes, two diagnostic features of conjunctivitis. Moreover, the data presented in Example 2 reveal that the protective/preventive effect conferred by FS-HBP2 pre-treatment is long lasting (at least 8 hours).

As shown in Example 3 of Exhibit B, the "Pre VAC life" or "Pre-MS-HBP1" readout (i.e., nasal secretion and nasal airway resistance prior to the administration of MS-HBP1) was considerably higher than the "Post VAC life" readout. This clearly shows that MS-HBP1 is capable of preventing allergic rhinitis because, despite the administration of histamine 15 minutes subsequent to the administration of MS-HBP1 life, nasal secretions and airway resistance were considerably reduced as compared to those of subjects to whom only histamine was administered (Pre VAC life measurements).

Example 1 of Exhibit C demonstrates that administration of FS-HBP2 (referred to in Exhibit C as EV131) prior to antigenic challenge reduces the symptoms/responses characteristic of allergic asthma and acute respiratory distress syndrome (ARDS) dramatically. Example 2 of Exhibit C reveals the efficacy of FSH-BP2 (EV131) in the treatment of allergic conjunctivitis.

In that D.RET6 is related by **both** structure and biological function to FS-HBP2 and MS-HBP, these results underscore the likelihood that D.RET6 also shares these preventive and therapeutic properties. Thus, Applicants assert that the evidence presented in the specification, in combination with the results presented in Exhibits A-C attached hereto, renders apparent to a skilled practitioner how to utilize D.RET6 in the prevention of an allergic reaction or inflammatory condition.

In view of the above, applicants contend that the rejection of claims 25-28, 30-34, and 39-44 under 35 U.S.C. §112 is inappropriate and respectfully request that the rejection be withdrawn.

Claims 1, 4, 6, 10, 18-34, and 39-44 have been rejected under 35 USC § 112, first paragraph, for containing subject matter which was allegedly not described in the specification in such a way as to convey that the inventors were in possession of the claimed invention at the time of filing. In view of Applicants arguments presented herein and the amendments to the claims, the rejection, as it applied to claims 1, 4, 6, 10, 18-34,

and 39-44, is respectfully traversed.

Claims 1, 4, 6, 10, 18-34, and 39-44 have been rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. Claims 19, 33, 39-40, 41, and 43 are canceled herein, thereby obviating any rejection of these claims.

Claims 1 and 4 and dependent claims therefrom are amended herein to delete the phrase "substantially the same". Claims 4 and 6 have been amended to reflect the appropriate numbering of amino acid residues within the full length D.RET6 protein which includes the 17 amino acid signal sequence. Amendments to these claims render the number designations of D.RET6 amino acid residues consistent throughout the claims and sequence listing, thereby adding clarity to the claims. Specifically, claim 4 is amended to refer to residue 131 of SEQ ID NO: 4, and claim 6 is amended to refer to residues 179 and 151 SEQ ID NO: 4. Claim 20 has been amended to replace the term "effector molecule" with "a toxin or bioactive molecule". Applicants assert that the term a toxin or bioactive molecule is definite and well defined at page 11, lines 2-20 of the specification. Claims 25-26 are amended to replace the phrase "associated with" with "fused to", which is a term well understood in the art. Claim 29 is amended herein to delete reference to the narrow range previously recited therein.

In view of the amendments to claims 1, 4, 6, 10, 18, 20-32, 34, 42, and 44, Applicants assert that the rejection of these claims on the basis of alleged indefiniteness is hereby nullified. Applicants, therefore, respectfully request that the Examiner withdraw the rejection of claims 1, 4, 6, 10, 18, 20-32, 34, 42, and 44 under 35 U.S.C. §112, second paragraph.

Rejections under the judicially created doctrine of obviousness-type double patenting

The Examiner has rejected claims 1, 4, 19, 21-22, 24, 29-30, 33-34, 39, and 41-44 under the judicially created doctrine of obviousness-type double patenting as allegedly unpatentable over claims 1-13 of U.S. Patent No. 6,617,312. A Terminal Disclaimer is attached hereto, the filing of which is believed to overcome the above rejection of pending claims 1, 4, 21-22, 24, 29-30, 34, 42, and 44 of the present invention under the judicially created doctrine of obviousness-type double patenting.

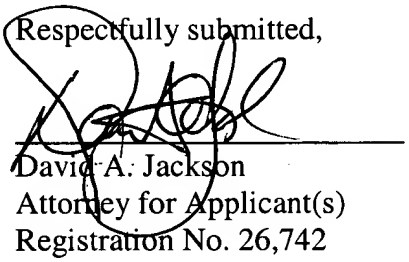
Fees

No additional fees are believed to be necessitated by this amendment. However, should this be an error, authorization is hereby given to charge Deposit Account No. 11-1153 for any underpayment or to credit any overpayment.

Conclusion

It is submitted, therefore, that the claims are in condition for allowance. No new matter has been introduced. Allowance of all claims at an early date is solicited. In the event that there are any questions concerning this amendment, or application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

Respectfully submitted,



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Enclosures: Petition for a Three-Month Extension of Time
Exhibits A, B, and C and Figure sets described therein
Terminal Disclaimer

Exhibit A

Serotonin binding properties of D.RET6

To demonstrate the binding of D.RET6 to serotonin, the following experiment was conducted:

Method:

Immortalised human carcinoid cells from the BON-1 cell line were cultured in 4ml of medium at a density of 200,000 cells per dish. These cells are known to secrete serotonin under these incubation conditions. Supernatant was collected after 48 hours and serial dilutions (1,000, 10,000 and 100,000 fold) were prepared. 25μl of D.RET6 from each of three prepared solutions (10.8mg/ml, 11.2mg/ml and 11.3mg/ml) was added to each of the dilutions of supernatant which were then analysed for serotonin. Undiluted supernatant and samples of each dilution to which D.RET6 had not been added were used as controls.

Results:

Dilution	**Control**	**D.RET6 10.8mg/ml**	**D.RET6 11.2mg/ml**	**D.RET6 11.3mg/ml**
Undiluted	43 nmol/L	-	-	-
x 1,000	60 nmol/L	0 nmol/L	0 nmol/L	0 nmol/L
x 10,000	232 nmol/L	0 nmol/L	0 nmol/L	0 nmol/L
x 100,000	204 nmol/L	0 nmol/L	0 nmol/L	0 nmol/L

Serotonin levels in supernatant from BON-1 cell incubation

Conclusion:

This experiment showed that D.RET6 rendered serotonin undetectable in the assay used.

Exhibit B

Example 1

In this study, the irritability and efficacy of various concentrations of an ophthalmic solution of a vasoactive amine binding protein have been evaluated in a compound 48/80 model of mast cell degranulation in the rabbit.

Compound 48/80 is the condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde, and promotes the release of allergy mediators, including histamine, from the mast cell. Due to its pro-inflammatory actions, Compound 48/80 has been used to screen new anti-allergic compounds in animals (Udell *et al.*, Am. J. Ophthalmol., 91, (2), 226-230, 1981).

In the present study, Compound 48/80 was used to determine the efficacy of the vasoactive amine binding protein FS-HBP2 as described in European patent application EP 98955786.3 (herein referred to by its internal designation "EV131" and in the Figures by the designation "HBP") in preventing the signs of allergic conjunctivitis. Conjunctivitis, or "pink eye", is the name given to the inflammation of the conjunctiva of the eye when exposed to bacteria, viruses or other irritants. Conjunctivitis is the most common eye disease in the developed world and can vary in severity from a mild inflammation with tearing to a severe inflammation that causes tissue injury.

EV131 ophthalmic solution was prepared in 1% and 6% concentrations from stock that contained approximately 2 mg EV131 and 50 microliters dH_20. Physiological saline, pH 7.2, was used as the buffer to make the dilutions.

Treatment was with either saline, or with 1%, 6% or 10% EV131 using the rabbit model. Each rabbit was topically dosed in the right eye with 40 microliters EV131 solution, and in the left eye with 40 micoliters saline.

Rabbits were given a baseline gross examination for hyperaemia, chemosis, mucous discharge and lid swelling. One rabbit that showed an abnormal examination (>+1 hyperaemia and corneal changes) was excluded from the study.

Five rabbits were dosed with 1% EV131 and four rabbits were dosed with 6% EV131. Ten minutes following dosing, 25 microliters of a 7.5 mg/ml of a solution of Compound 48/80 (Sigma Chemical Co., St. Louis, MO, USA) was topically instilled in the pre-dosed eyes.

All rabbits were examined by gross examination at 3 min, 5 min, 10 min, 20 min, 60 min, 8 hours and 24 hours following challenge with Compound 48/80. Eyes were evaluated for conjunctival injection, chemosis, tearing, mucous discharge and lid swelling.

A dose of 6% (97μg) EV131 was found to give optimum results of consistent reduction in inflammation as measured by hyperaemia, chemosis, mucous discharge and lid swelling.

After a three week refractory period, the procedure was repeated. This time, four rabbits were dosed with 6% EV131 and five rabbits were dosed with 10% EV131.

One rabbit in the 6% EV131 group had mucus in both eyes. Three rabbits in the 6% group (1 rabbit at 20, 60 and 240 minutes post-challenge and 2 rabbits at 60 minutes post-challenge) had mucus only in the placebo pre-treated eye. In the 10% treated group, 1 rabbit only had mucus in the EV131 treated eye (60 minutes and 8 hours post-challenge) and three rabbits had mucus in both eyes (60 minutes and 8 hours post-challenge).

The data are displayed in Figures 1 to 5. Figure 1 shows the mean change from baseline redness scores following pre-treatment of rabbit eyes with either 1% EV131 (HBP) or saline. Figure 2 shows the mean change from baseline redness scores following pre-treatment of rabbit eyes with either 6% EV131 (HBP) or saline. Figure 3 shows the mean change from baseline redness scores following pre-treatment of rabbit eyes with either 10% EV131 (HBP) or saline. Figure 4 shows the mean change from baseline chemosis scores following pre-treatment of rabbit eyes with either 6% EV131 (HBP) or saline. Figure 5 shows the mean change from baseline chemosis scores following pre-treatment of rabbit eyes with either 10% EV131 (HBP) or saline.

Example 2

This work concerned a study of the effect of dosing EV131 in the rabbit eye for an extended period, before 48/80-induced inflammation was assessed.

Eight rabbits were used, the right eye being used for test material and the left for saline control. Each rabbit underwent a 7 day loading with EV131, 40µl of 6% solution once daily, equivalent to a total daily dose of 96.0µg of EV131. 40µl of physiological saline (pH 7.2) was instilled into the left (control) eye at the same time. Eight hours following the final dose of test and control materials 25µl of substance 48/80 (7.5mg/ml) was instilled into each eye. Observations and scoring of eye redness were taken at 3, 10, 20 and 60 minutes and 8 hours post instillation of 48/80. Mean values were calculated and are shown in Figure 6, which shows the mean change from baseline redness scores following one week loading with either 6% EV131 or saline and then challenge with 48/80 8 hours following dosing.

The results show that at 3 minutes, there was a mean 65.0% reduction in eye redness compared to control in the EV131 eye, at 5 minutes a 33.3% reduction and at 10 minutes a 40.6% reduction. At 20 minutes there was a mean 5% increase in eye redness in the test eye compared to control. At 60 minutes and 8 hours there was no difference in eye redness between test and control eyes.

These results show that eight hours following the final dose of test and control materials, there was evidence of a protective effect of EV131 compared to saline control in reducing the degree of eye redness following instillation of substance 48/80. This effect persisted for at least 10 minutes but was not apparent at 20 minutes (i.e. 8 hours and 20 minutes following the final dose of EV131). This shows evidence of duration of action against 48/80 induced degranulation of conjunctival mast cells of at least 8 hours.

Example 3

Allergic rhinitis is the medical term given to the inflammation of the nasal mucosa caused by allergens such as pollen or dust. There are two general types of allergic rhinitis, seasonal and perennial. Seasonal allergic rhinitis is

normally referred to as hay fever and is usually caused by a reaction to mold or pollen. Perennial allergic rhinitis is usually caused by an inherent sensitivity to one or more types of allergen. This condition generally continues throughout the year or for as long as the patient is exposed to the allergen. The condition is thought to affect more than 15% of the population of the western world.

In this study, three subjects were challenged intranasally with histamine. The histamine concentrations used were 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 4.0 mg/ml and, where necessary to achieve a 100% or greater increase in nasal airway resistance on the pre-treatment challenge, 8 mg/ml. One hundred microliters of each dose was administered to each nostril for each challenge.

Initially, baseline measurements were taken of the subjects' anterior nasal secretions. Nasal secretions were measured by asking subjects to blow their noses into pre-weighed paper handkerchiefs and then re-weighing them to calculate the weight of secretions produced.

Each subject was then administered a nasal histamine dose-response challenge. Forty five minutes after the completion of the challenge, baseline measurements were repeated. Then a histacalin protein, MS-HBP1 as described in European patent application EP 98955786.3 (referred to internally as EV504), was administered as a fresh solution of pre-weighed aliquots of histacalin in phosphate buffered saline. The solution was administered by dropping from a pipette into each nostril. In the attached Figures 7, 8, 9 and 10, it is referred to as VAC life or Histamine binding protein.

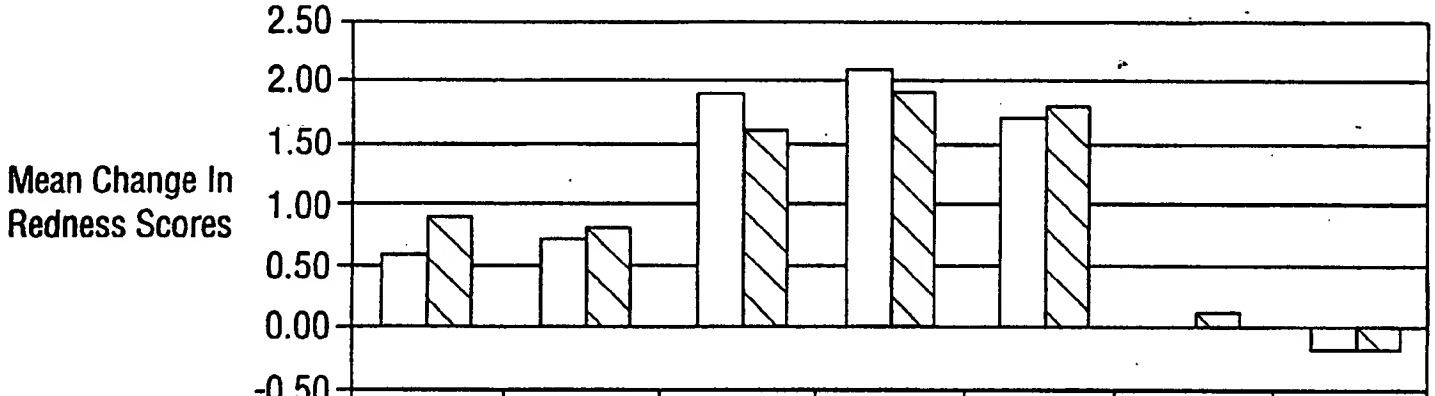
After a further 15 minutes, a repeat nasal histamine dose-response challenge was administered. The outcome measurements are recorded as total nasal airway resistance, as measured by active posterior rhinomanometry (placing inflatable balloons in the posterior nares and monitoring changes in pressure and volume), and by measurement of anterior nasal secretions, as measured by weight of expelled secretions. The anterior nasal secretions are represented as a cumulative total for the histamine challenges. These measurements are shown in Figures 7, 9a, 9b and 9c. Figure 7 shows a table of the data obtained for three volunteer subjects relating to nasal secretions. Figures 9a, 9b and 9c depict the data pertaining to nasal secretions in graphic form for each individual subject.

For Figures 8, 10a, 10b and 10c, the nasal airway resistance measured has been represented as a percent change from a saline challenge response (undertaken as the first challenge in the histamine dose-response challenge). Figure 8 shows a table of the data obtained from three volunteer subjects relating to nasal airway resistance. Figures 10a, 10b and 10c show the data for nasal airway resistance in graphic form for each individual subject.

In this study, it was concluded that MS-HBP1 caused a mean 4-fold shift in the dose response to histamine. These data indicate that MS-HBP1 is an excellent candidate therapeutic agent for the prevention and treatment of nasal allergic rhinitis.

FIG. 1

Mean Change From Baseline Redness Scores Following Pre-Treatment With Either 1% HBP Or Saline

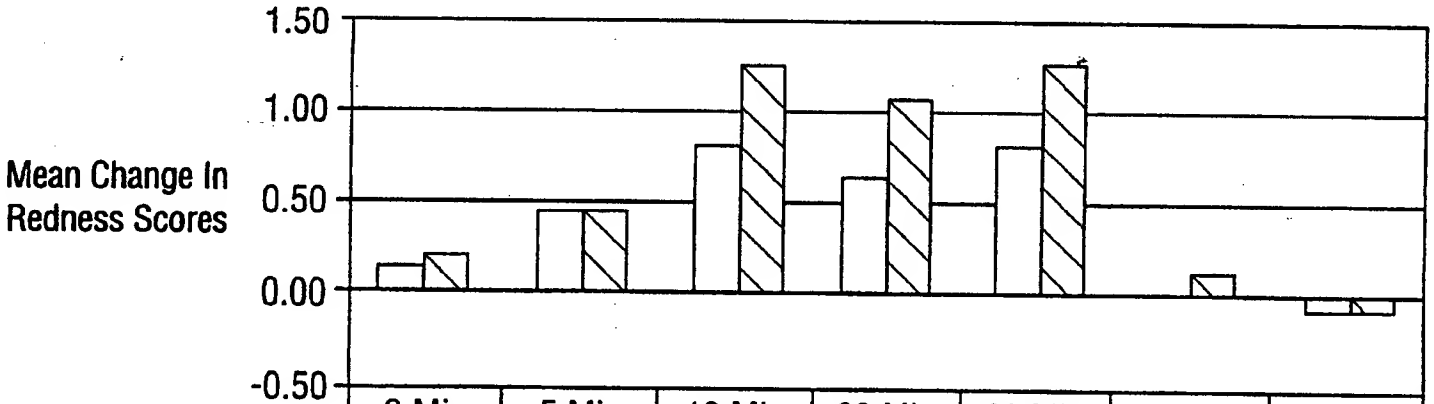


	3 Min	5 Min	10 Min	20 Min	30 Min	60 Min	8 Hrs
☐ HBP 1%	0.60	0.70	1.90	2.10	1.70	0.0	-0.2
▨ Saline	0.90	0.80	1.60	1.90	1.80	0.1	-0.2

Time Post Challenge with Compound 48/80 (min)

FIG. 2

Mean Change From Baseline Redness Scores Following Pre-Treatment With Either 6% HBP Or Saline

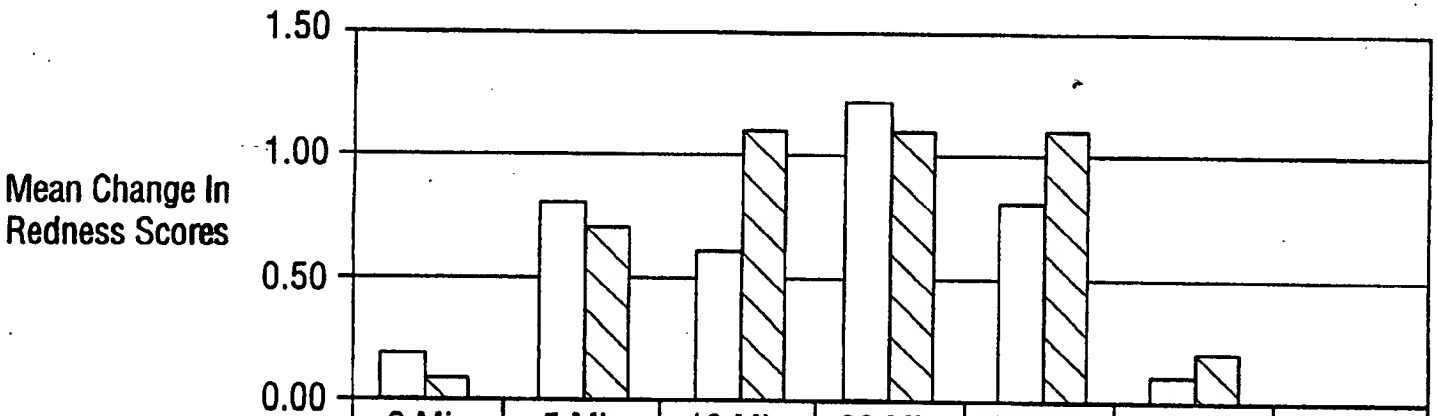


	3 Min	5 Min	10 Min	20 Min	60 Min	8 Hrs	24 Hrs
☐ HBP 6%	0.13	0.44	0.81	0.63	0.81	0.00	-0.06
▨ Saline	0.19	0.44	1.25	1.06	1.25	0.13	-0.06

Time Post Challenge with Compound 48/80 (min)

FIG. 3

Mean Change From Baseline Redness Scores Following Pre-Treatment With Either 10% HBP Or Saline

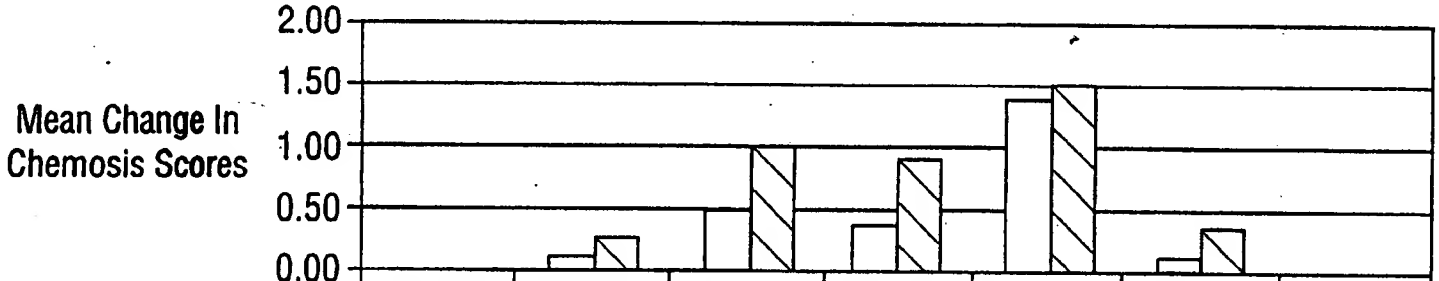


	3 Min	5 Min	10 Min	20 Min	60 Min	8 Hrs	24 Hrs
☐ HBP 10%	0.20	0.80	0.60	1.20	0.80	0.1	0.0
▨ Saline	0.10	0.70	1.10	1.10	1.10	0.2	0.0

Time Post Challenge with Compound 48/80 (min)

FIG. 4

Mean Change From Baseline Chemosis Scores Following Pre-Treatment With Either 6% HBP Or Saline

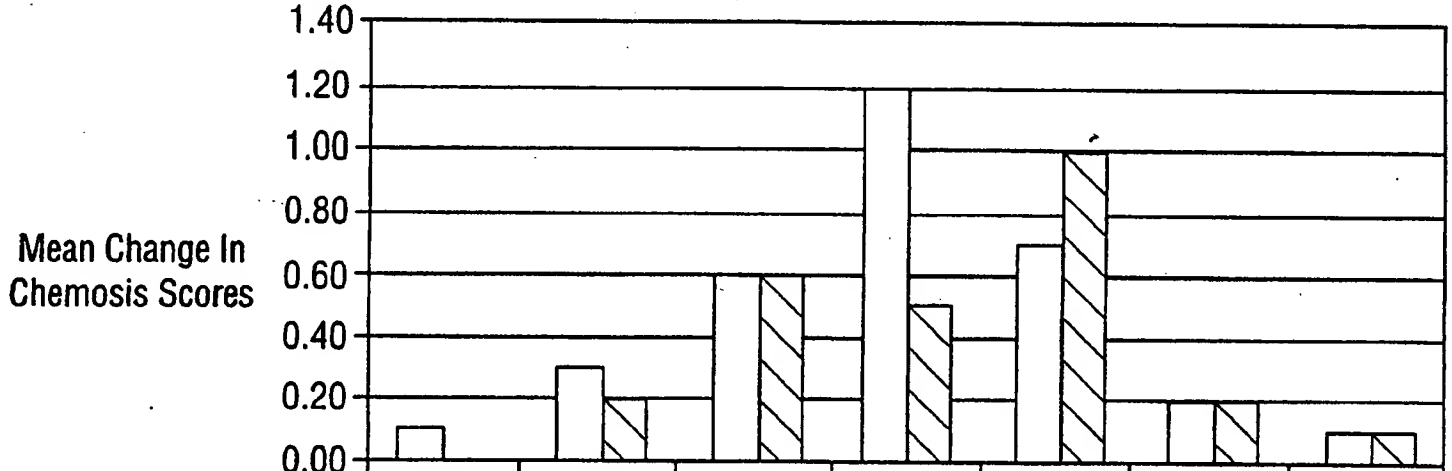


	3 Min	5 Min	10 Min	20 Min	60 Min	8 Hrs	24 Hrs
HBP 6%	0.00	0.13	0.50	0.38	1.38	0.13	0.00
Saline	0.00	0.25	1.00	0.88	1.50	0.38	0.00

Time Post Challenge with Compound 48/80 (min)

FIG. 5

Mean Change From Chemosis Scores Following Pre-Treatment With Either 10% HBP Or Saline

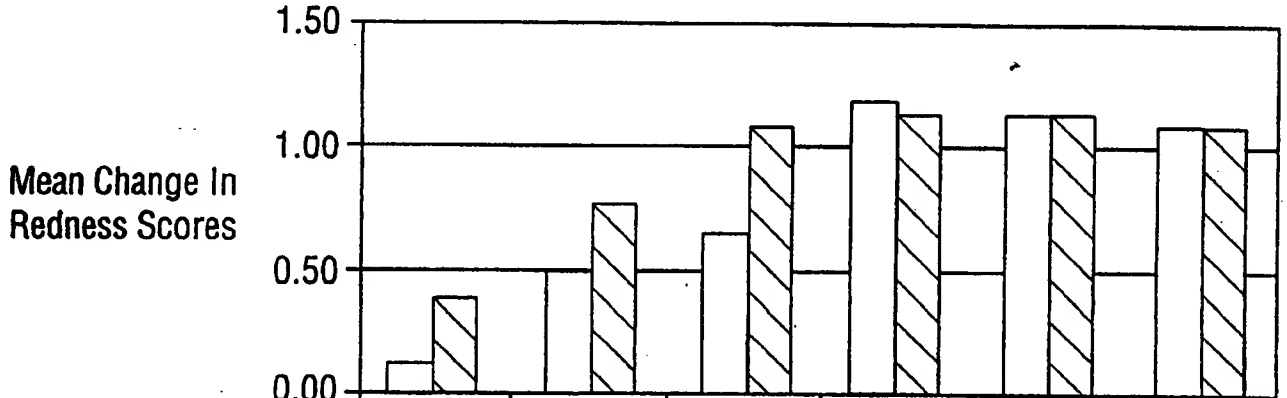


	3 Min	5 Min	10 Min	20 Min	60 Min	8 Hrs	24 Hrs
☐ HBP 10%	0.10	0.30	0.60	1.20	0.70	0.2	0.1
▨ Saline	0.00	0.20	0.60	0.50	1.00	0.2	0.1

Time Post Challenge with Compound 48/80 (min)

FIG. 6

Mean Change From Baseline Redness Scores Following One Week Loading With Either 6% HBP (EV131) Or Saline And Then Challenge with 48/80 8 Hours Following Dosing



	3 Min	5 Min	10 Min	20 Min	60 Min	8 Hrs
☐ HBP (EV131) 6%	0.13	0.50	0.63	1.19	1.13	1.06
▨ Saline	0.38	0.75	1.06	1.13	1.13	1.06

Time Post Challenge with Compound 48/80 (min)

FIG. 7

Vacs of Life Study: Histamine binding protein
Histamine Nasal Challenge
Weight of Anterior Nasal Secretions (grams)

	Histamine (mg/ml)				
Subject	0.5	1.0	2.0	4.0	8.0
PH					
Pre	0.023	0.022	0.294	0.456	----
Post	0.095	0.077	0.071	0.229	----
JW					
Pre	0.451	0.323	0.303	0.286	----
Post	0.060	0.060	0.238	0.133	0.328
KC					
Pre	0.454	0.378	0.374	0.579	0.175
Post	0.245	0.288	0.236	0.249	----

FIG. 8

Vacs of Life Study: Histamine binding protein
Histamine Nasal Challenge
Nasal Airways Resistance (kPa/L/sec)

				Histamine (mg/ml)				
Subject	B	PVL	PS	0.5	1.0	2.0	4.0	8.0
PH								
Pre	0.193	----	0.181	0.203	0.498	0.461	0.543	----
Post	0.235	0.185	0.190	0.187	0.250	0.297	0.317	----
JW								
Pre	0.174	----	0.162	0.182	0.204	0.242	0.414	----
Post	0.131	0.172	0.223	0.228	0.254	0.287	0.444	0.538
KC								
Pre	0.190	----	0.184	0.211	0.245	0.314	0.321	0.960
Post	0.207	0.214	0.228	0.245	0.288	0.236	0.249	0.843

B = Baseline PVL = post Vacs of Life PS = Post saline

FIG. 9a

Nasal histamine challenge on subject PH

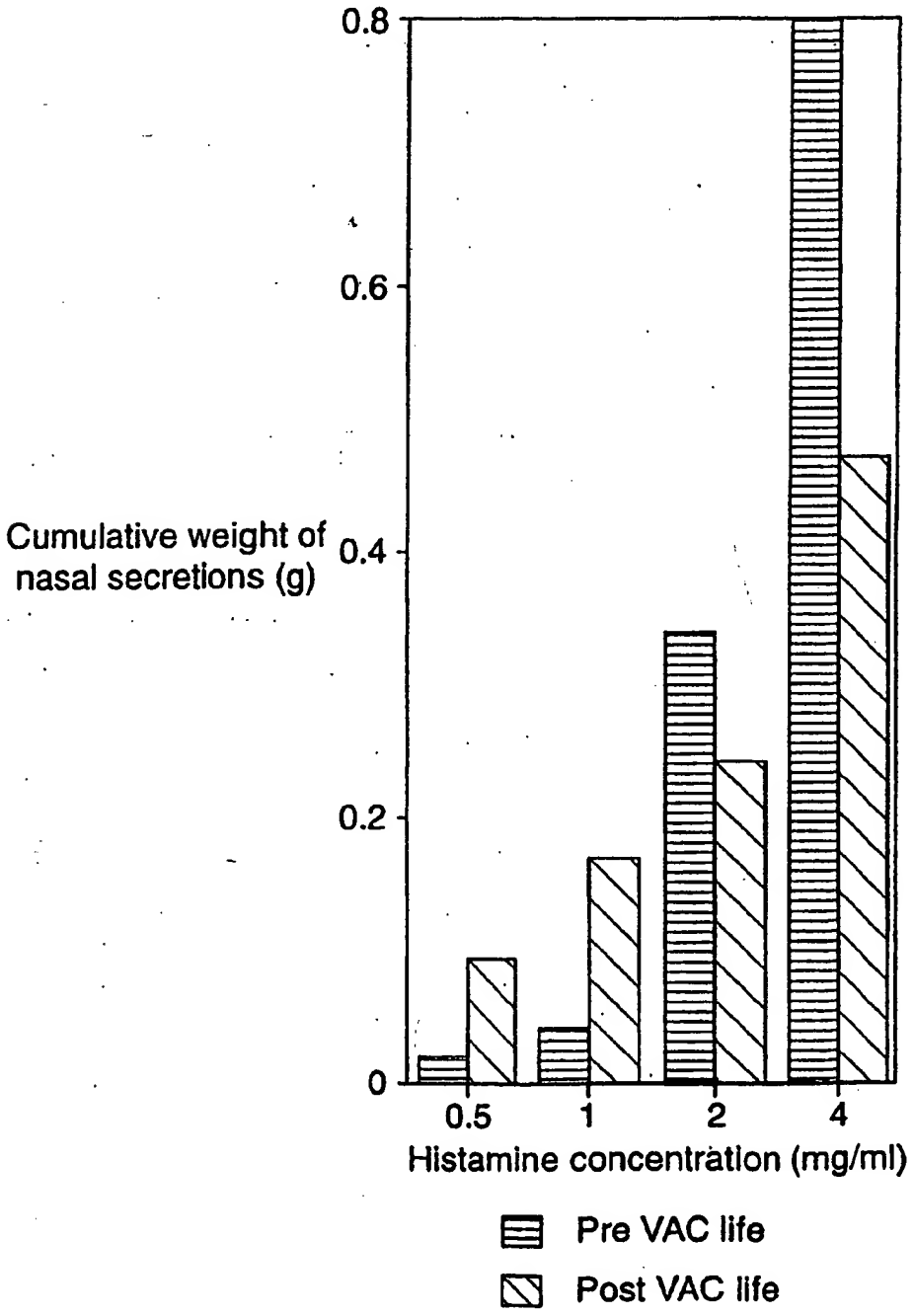


FIG. 9b

Nasal histamine challenge on subject JW

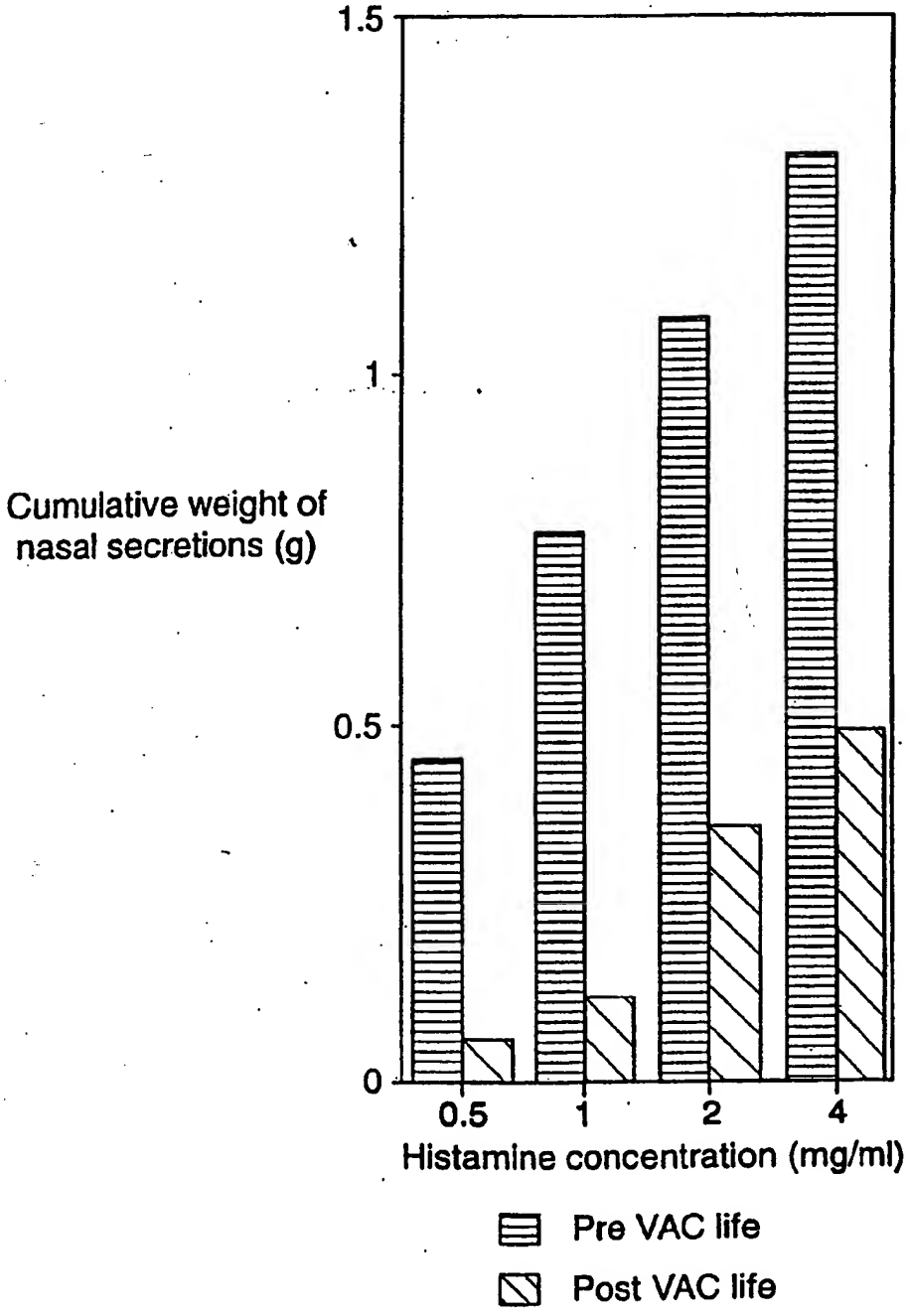


FIG. 9c

Nasal histamine challenge on subject KC

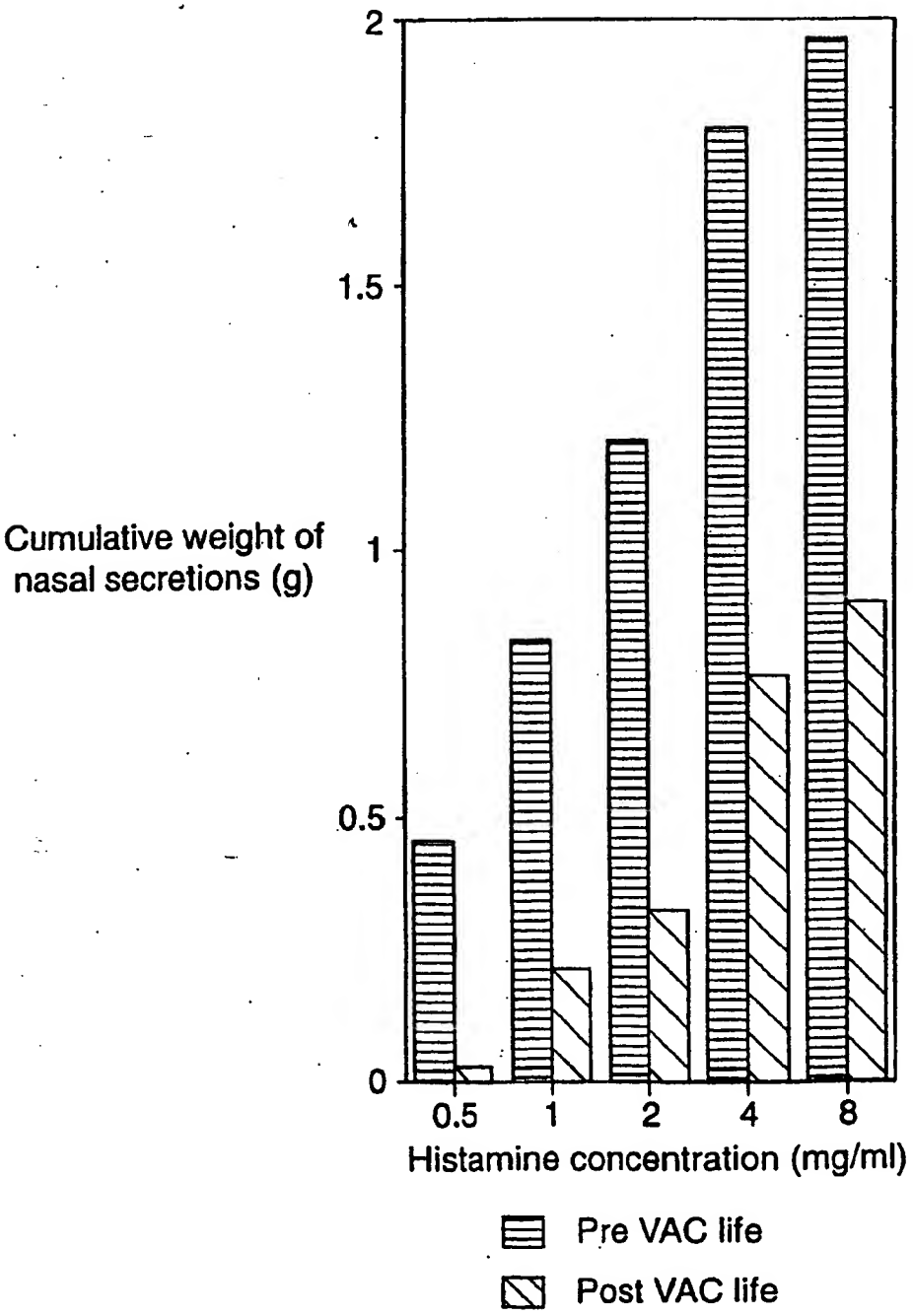


FIG. 10a

Nasal histamine challenge on subject PH

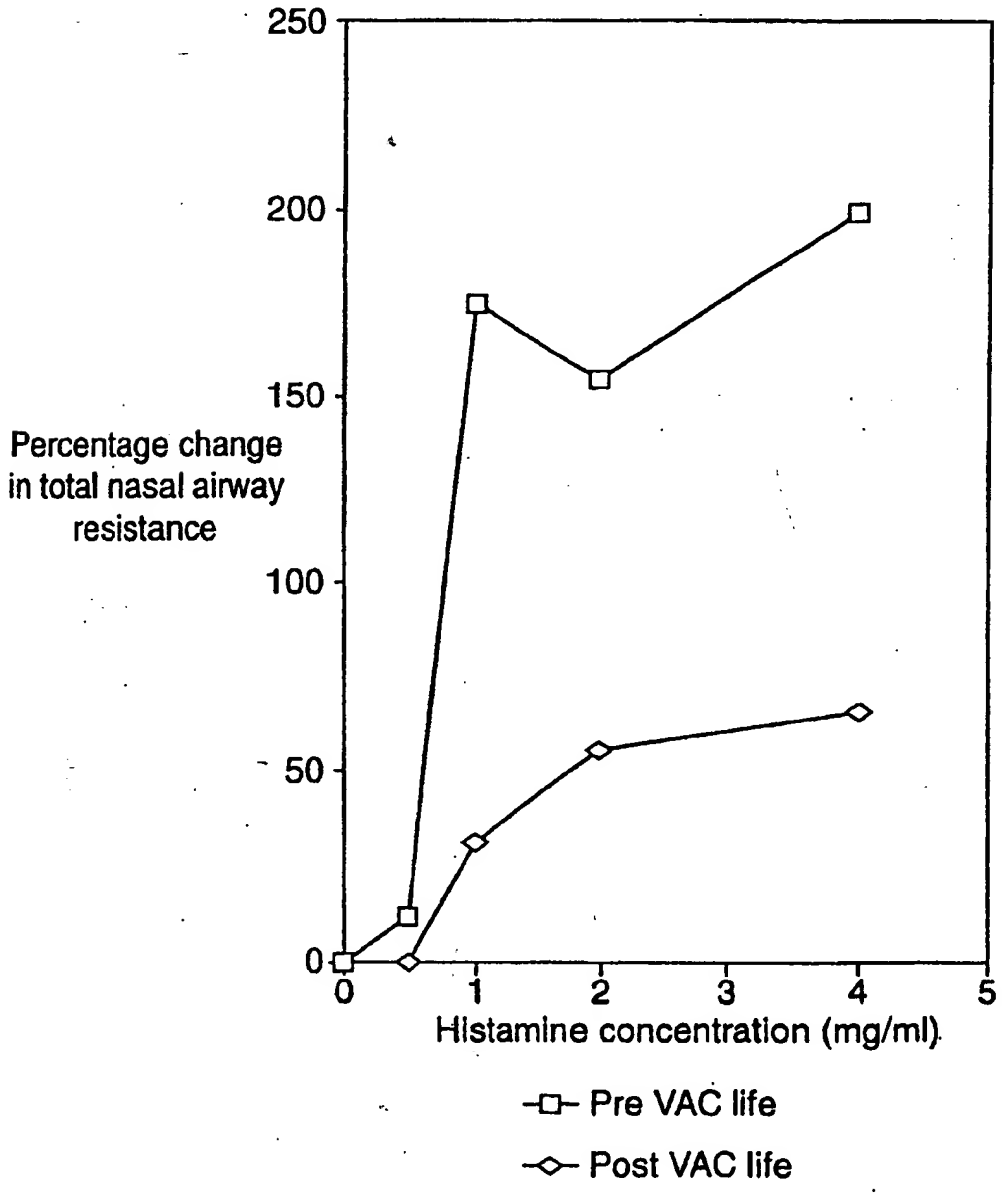


FIG. 10b

Nasal histamine challenge on subject JW

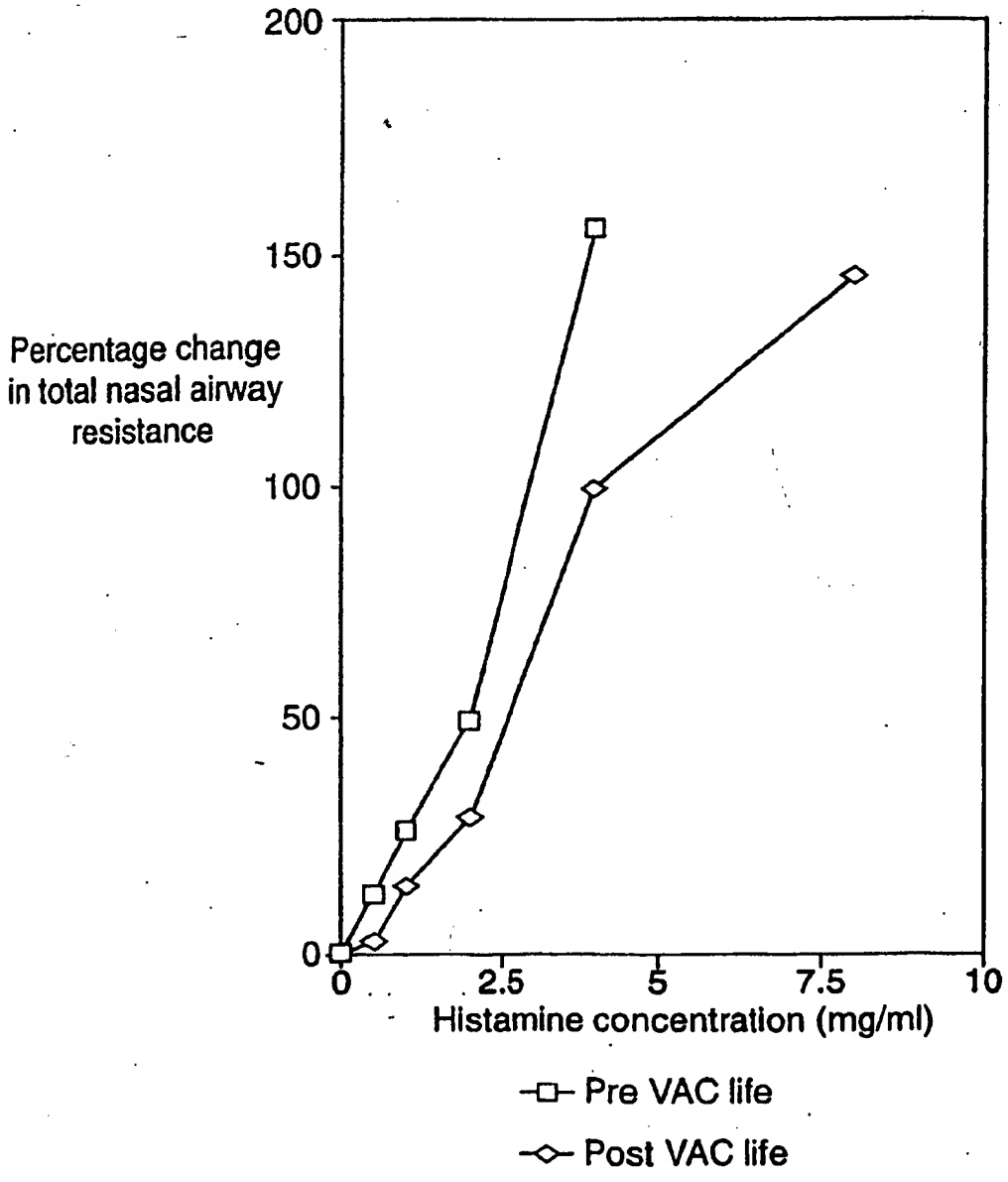


FIG. 10c

Nasal histamine challenge on subject KC

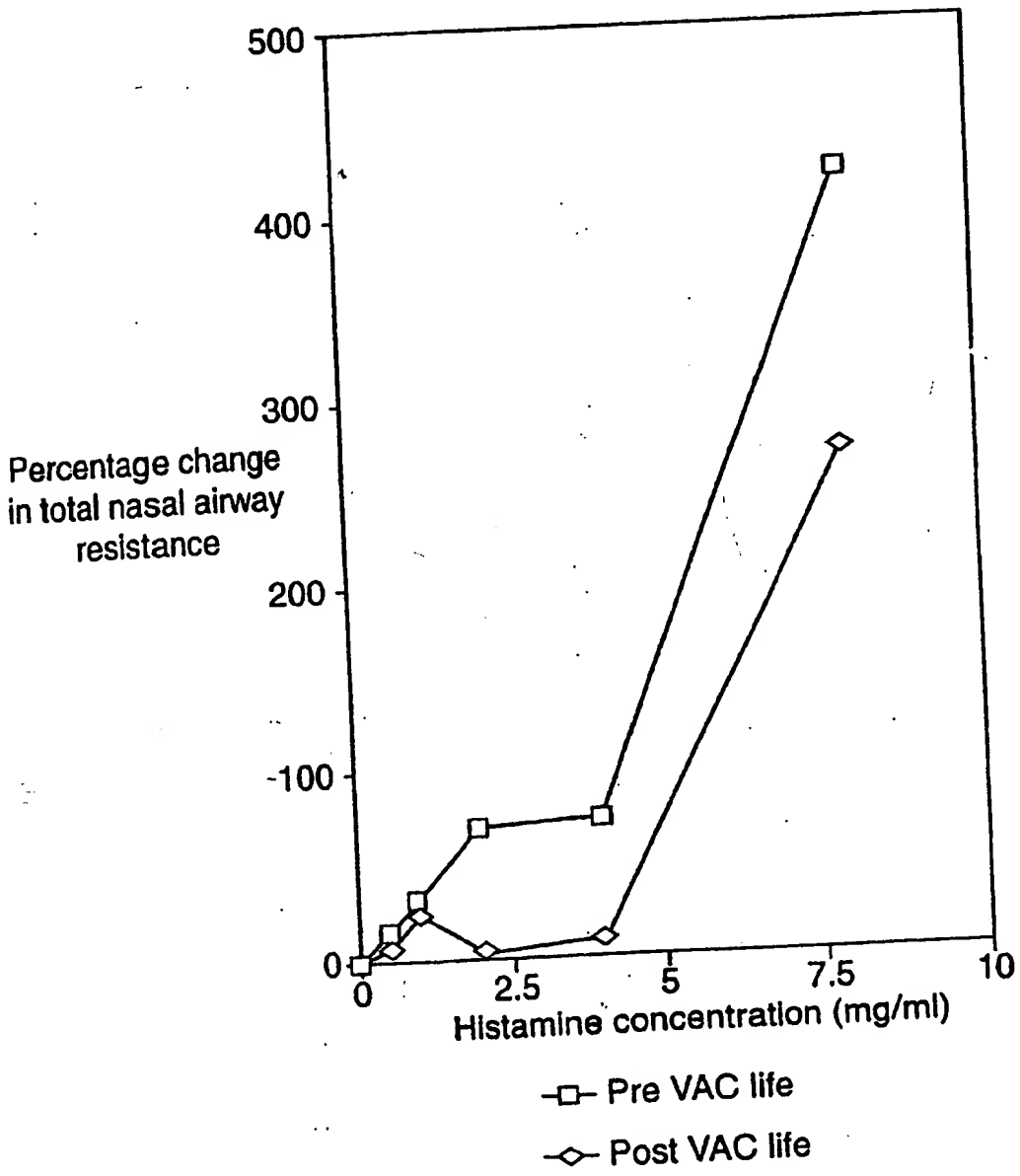


Exhibit C

Example 1: Allergic asthma

The recombinant, arthropod derived histamine binding protein EV131 binds histamine with high affinity (Paesen, G. C., P. L. Adams, K. Harlos, P. A. Nuttall, and D. I. Stuart. 1999, Mol Cell 3:661; Paesen, G. C., P. L. Adams, P. A. Nuttall, and D. L. Stuart. 2000, Biochim Biophys Acta 1482:92).

Initially, tests were performed to ascertain whether EV131 might inhibit pathologies mediated by histamine. EV131 was therefore tested in allergic asthma. EV131 given prior to antigen challenge in immunised mice was found to prevent airway hyperreactivity by 70%, abrogated peribronchial inflammation, pulmonary eosinophilia, mucus hypersecretion and IL-4 secretion (Couilllin *et al*, submitted). The inhibitory effect of EV131 on bronchial hyperreactivity was comparable to that of glucocorticosteroids. These results demonstrate that histamine is a critical mediator of allergic asthma.

The results of these tests prompted us to investigate acute respiratory distress syndrome (ARDS), which shows certain features in common with allergic asthma. A model for ARDS was established using a single administration of *E. coli* endotoxin (Lefort, J., L. Motreff, and B. B. Vargaftig. 2001, Am J Respir Cell Mol Biol 24:345.). It is here shown that EV131 dramatically inhibits bronchoconstriction and neutrophil recruitment.

Methods

Induction of acute bronchoconstriction by E. coli endotoxin

The optimal dose of endotoxin that would produce maximal airways responses without killing the mice was first established using saline alone as control. This was determined to be 1 μg (data not shown). *E. coli* endotoxin (055:B5, Sigma) was dissolved in saline and given to C57BL/6 mice at a dose of 1 μg in 40 μl saline via the intranasal route under i.v. ketamine anaesthesia (to prevent coughing). rEV131 was given at three dose levels (90, 180 and 360 μg, 4.5 – 18 mg/Kg) to different groups of mice immediately before endotoxin by the same route, controls received saline only.

In a second set of experiments using this model, 350μg budenoside (positive control), saline (negative control) and 182μg rEV131 were given intraperitoneally by injection, one hour before the 1μg LPS dose, which again was given by nasal inhalation.

Airways resistance: Plethysmography

The airway resistance was evaluated by whole-body plethysmography for 3h after endotoxin administration. After a recovery period bronchial hyperreactivity (BHR) to aerosolized methacholine was then investigated. Unrestrained conscious mice who had received endotoxin and either active or control medication were first placed in whole-body plethysmography chambers (Buxco Electronic, Sharon, CO, USA). The mouse is placed in one of two barometric plethysmography chambers linked to suction pumps that ensure constant airflow. The animal is introduced into the first chamber separated from the second in which pressure corresponds to atmospheric pressure. Each compartment is linked to two parts of a differential pressure captor, which is itself connected to an electronic amplifier and signals are analyzed by software. This system allows the quantification of many parameters during successive respiratory cycles. Using this system bronchoconstriction was evaluated for three hours using Enhanced Respiratory Pause (Penh) as an indicator of airways resistance.

Penh can be conceptualised as the phase shift of the thoracic flow and the nasal flow curves; increased phase shift correlates with increased respiratory system resistance. Penh is calculated by the formula Penh = (Te/RT-1) x PEF/PIF, where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow. Penh values correspond to the mean of eleven events (cycles) every five seconds during the observation period.

The experiment was terminated after 180 minutes and mice were allowed to recover by being ventilated with high oxygen concentration before being investigated for residual BHR.

In this phase of the experiment methacholine at 300 mM was aerosolised and introduced into the plethysmograph chambers for 20 seconds and mean airway bronchoconstriction readings, as assessed by Penh, were obtained over a 15-min period, which is the duration of methacholine induced BHR.

After analysis of data, Penh values are shown in Fig. 1 for 36 time points after endotoxin administration and 5 time points after methacholine nebulization. Penh values at every point correspond to the mean of Penh values between 5 min before and 3 min after the point. (*NB In Fig. 1 the recovery period corresponds to the apparent drop in Penh at 180 minutes*).

Bronchoalveolar lavage (BAL)

BAL was performed under strong ketamine and xylasine anaesthesia 3.5h after intranasal endotoxin administration by rinsing the airways with 4 volumes of 0.5ml each of ice-cold phosphate buffered saline (PBS). The lavage fluid was centrifuged, resuspended, total cells were counted using a haematocytometer chamber and cytospin preparations were prepared using a Shandon cytocentrifuge. The cells were analysed: after differential staining with May-Gruenwald-Giemsa.

Myeloperoxidase assay of the lung (MPO)

In order to assess the neutrophil content in the lung we analysed the amount of myeloperoxidase, a major enzyme of neutrophils, in the lung as described before (also, see Hoy A, Leininger-Muller B, Kutter D, Siest G, Visvikis S. Clin Chem Lab Med 2002; 40(1): 2 – 8.)

Lung histology

After bronchoalveolar lavage, the mice were killed. The whole lung was removed and fixed in 4% buffered formaldehyde for standard microscopic analysis using H&E stain. The peribronchial infiltrate and the smooth muscle hyperplasia was assessed by a semi-quantitative score (0 – 3) by two independent observers.

Results

Bronchoconstriction induced by endotoxin is inhibited by EV131

Firstly we established a dose-response effect of endotoxin (1-100 μg) that induced non-lethal bronchoconstriction. Endotoxin was found to induce a substantial bronchoconstriction within 15-30 min (data not shown). We selected a dose of 1 μg of endotoxin for the further experiments in order to test the effect of rEV131.

In the control group LPS induced a substantial bronchoconstriction that peaked at about 80 minutes and persisted for 180 minutes until the mice were allowed to recover in high oxygen conditions. When given intranasally at a dose of 360 μg rEV131 partially inhibited this response whilst, 90 μg and 180 μg had a greater inhibitory effect (Figure 1). This result was initially surprising, but subsequently, it was realised that the mouse given 360μg rEV131 was suffering from an infection and probably a neutrophilia pre-treatment, and

was thus not considered typical. Results from this mouse were excluded from subsequent analyses.

These data suggest that endogenous histamine plays a role in bronchoconstriction induced by endotoxin, and hence neutralisation of histamine by rEV131 could ameliorate ARDS.

Figure 4 shows a similar effect for rEV131 given intraperitoneally, proving that the rEV131 cannot be binding the LPS directly. This also demonstrates the rEV131 is effective when administered by this route.

Bronchial hyperreactivity (BHR) is inhibited by EV131

We also tested methacholine-induced BHR 3h after endotoxin administration and recovery in high oxygen conditions. First we demonstrated that methacholine-mediated BHR occurs following endotoxin administration as compared to saline control (data not shown). After this we investigated the effect of methacholine in the rEV131 dosed and control mice. Methacholine provoked bronchoconstriction in control mice but not in mice treated with rEV131 (Figure 1). Therefore, the data suggest that endotoxin-induced hyperreactivity is histamine dependent and can be attenuated by rEV131.

Reduced recruitment of neutrophils in BAL and lung

Administration of endotoxin results in a significant recruitment of neutrophils in BAL fluid. We recovered about 10^5 leukocytes in BAL fluid from control animals at 3h after endotoxin inhalation. Administration of rEV131 did not alter the total cell count in the BAL fluid but, in contrast, the recruitment of neutrophils was reduced by rEV131 at 180 and 90 μg although this did not reach statistical significance ($p< 0.2$). The 360 μg dose had no effect (Figure 2); however, only one animal was evaluated at this dose and as stated above, this animal was subsequently identified as suffering from an infection.

We also investigated whether the recruitment of activated neutrophils into the lungs was altered. In order to quantify the neutrophil recruitment we tested MPO activity of fresh lung homogenate. This showed a significant reduction of neutrophil activity by rEV131 at 180 μg ($p < 0.05$) and 90 μg ($p < 0.01$) (Figure 3). In the infected mouse, administered 360 μg, there was no effect.

Figures 5, 6 and 7 are equivalent experiments performed to evaluate cell recruitment in BAL fluid when rEV131 and budenoside are given intraperitoneally. As is evident from these graphs, total cell numbers in BAL are significantly reduced by rEV131, and

neutrophils in particular. Furthermore, the amount of TNF in the BAL fluid is also reduced by rEV131 (see Figure 8).

Lung histopathology:

Lungs from mice that received endotoxin showed significant peribronchial cellular infiltrates with abundant neutrophils (data not shown). rEV131 reduced the recruitment of neutrophils substantially at 180 μg and 90 μg doses (data not shown).

Conclusion

The present data demonstrate that the histamine binding protein rEV131 significantly inhibits endotoxin-induced bronchoconstriction, BHR and neutrophil recruitment in a murine model of ARDS. This effect is evident both when administered intranasally and intraperitoneally.

Example 2: Allergic conjunctivitis

A study was performed to evaluate the safety and efficacy of FS-HBP2 (rEV131) in the prevention of the signs and symptoms of allergic conjunctivitis as induced by the conjunctival allergen challenge model (Abelson MB, Chambers WA and LM Smith; Ophthalmology, 1990; 108:84-88). Four treatments were applied, involving a comparison of the rEV131 vehicle against three concentrations of rEV131 (0.06%, 0.12% and 0.24% ophthalmic solutions). Sixty subjects enrolled in the study.

Primary efficacy variables that were measured included ocular itching and redness. As part of the secondary efficacy variables measured, neutrophil counts were assessed. To do this, tear samples were collected from 23 subjects who participated in the study. Of those, 19 subjects had detectable neutrophil counts. In subjects that received the 0.12% (N=3) and 0.24% (N=7) concentrations of rEV131 in one eye and placebo in the fellow eye, neutrophil counts were significantly less in the drug-treated eye (see Figure 9). However, in the group that received 0.06% rEV131 in one eye and placebo in the other eye, neutrophil counts were significantly greater in the eyes that received medication. In the eyes receiving vehicle bilaterally (N=5), no significant difference in neutrophil counts were found.

These results suggest that unpreserved rEV131 may significantly decrease the number of neutrophils recruited to the eye during or immediately after the conjunctival allergen challenge.

These results, when combined with the results presented in Examples 1 and 2, suggest that unpreserved rEV131 (i.e. rEV131 solutions that do not contain the preservative benzalkonium chloride, with which the protein is suspected to complex) may play a more significant role in decreasing inflammation and subsequent tissue damage associated with more specifically, neutrophil-mediated diseases, in the acute allergic reaction in the eye. The late phase allergic reaction is mediated by the infiltrate of leukocytes into the tissue via chemotactic factors released by the mast cell during the early phase acute reaction. In the eye, while there may be a physiologic late phase with cellular infiltrate, most cases of allergic conjunctivitis do not have a clinically relevant late phase. In the eye, only severe, chronic allergic reactions consist of a late phase reaction which reaches a certain threshold and induces clinical signs and symptoms, such as keratitis and shield ulcers seen in vernal keratoconjunctivitis. However, the nose and lung do manifest clinical late phase reactions more prominently than the eye. This may explain the effects of rEV131 seen on nasal symptoms induced by conjunctival allergen challenge (CAC) in a previous clinical study. An allergic reaction in the nose, following CAC, and effects of an agent instilled in the eye, on nasal symptoms, is not unexpected since allergen, mediators, and active drug products, can all drain from the ocular surface, through the nasolacrimal ducts, into the inferior turbinate of the nasal cavity where it can elicit effects on nasal tissues.

Brief description of the figures

Figure 1: Endotoxin (LPS) induced bronchoconstriction and inhibition by EV131. LPS was given at 1mg by the intranasal route and EV131 at 360 μg, 180 μg and 90 μg. PenH values were measured for 3h. At 3 h the response to methacholine was analysed. The codes S01, S02 etc. each represent an individual mouse (souris).

Figure 2: EV131 inhibits endotoxin-induced neutrophil recruitment in BAL. LPS was given at 1μg by the intranasal route and EV131 at 360 μg, 180 μg and 90 μg. Total cells did not differ, while EV131 180 μg and 90 μg reduced the neutrophils in BAL.

Figure 3: EV131 inhibits endotoxin-induced neutrophil recruitment in lung as assessed by MPO activity. EV131 at 180 μg and 90 μg, but not at 360 μg inhibited MPO activity in the lungs.

Figure 4: Endotoxin (LPS) induced bronchoconstriction and inhibition by EV131 as administered intraperitoneally by injection. LPS was given at 1μg by the intranasal route and EV131 at 182 μg. PenH values were measured for 3h.

Figure 5: Total cell recruitment in BAL fluid when rEV131 and budenoside are given intraperitoneally.

Figure 6: Cell recruitment in BAL fluid when rEV131 and budenoside are given intraperitoneally, as differentiated by cell type.

Figure 7: Total cell recruitment in BAL fluid when rEV131 and budenoside are given intraperitoneally.

Figure 8: TNF in the BAL fluid is reduced by rEV131 as given intraperitoneally.

Figure 9: Neutrophil counts in tear samples suggest that unpreserved rEV131 significantly decreases the number of neutrophils recruited to the eye during or immediately after conjunctival allergen challenge in human patients.